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14. ABSTRACT

During this second year of research, we have modified patient recruitment protocols for a greater sample acquisition rate, and initiated studies in mouse models of prostate cancer. This will allow us to reduce the influence of genetic heterogeneity observed in human populations, and increase our confidence that observed differences in protein levels are the result of a malignant process. From collected patient samples, we now are able to isolate CD4+ T-lymphocytes and monocytes at high yield and high purity, and analyze them by mass spectrometry. We have observed a total of 1190 proteins from monocytes by two or more peptides from these samples. At this early stage, there are numerous proteins which could become candidates for discriminating between prostate cancer and benign prostate diseases in the setting of an elevated PSA. However, the main task in the coming year will be the analysis of a much larger number of samples, in order to achieve statistical significance.

15. SUBJECT TERMS
Prostate cancer, biomarker, disease classifier, proteomics, leukocyte, mass spectrometry

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INTRODUCTION: This research has the long-term goal of developing a test that will discriminate between prostate cancer (PrCA) and benign prostate disease. The PSA test is sensitive for prostate cancer but not specific – many elevated levels of PSA are associated with the diseases of chronic prostatitis (CP) or benign prostatic hyperplasia (BPH) [4]. If, after an elevated PSA test, our test could distinguish among these possibilities, we could better take advantage of the sensitivity of the PSA test, with the enhanced specificity resulting from our test. Among other benefits, this would be significant for reducing the need for prostate biopsies, with all its attendant costs and potential morbidities [5,6,7]. Our approach is to look for biomarkers present on circulating leukocytes that will distinguish among the three conditions. We hypothesize that it is likely such differences exist because of the very different natures of the inflammatory response in those conditions [8,9]. The practical rationale for this approach is that circulating leukocytes are present in the easily accessible clinical specimen of blood, yet avoids the pitfalls of serum proteomics, in which highly abundant proteins make the identification of biomarkers extremely challenging [10]. More broadly, our proposed studies will add to our understanding of the complex relationship among inflammation, the immune system and the development of prostate cancer. This knowledge may aid in the earlier identification of cases destined to become clinically significant cancers, as opposed to those which may be present are not destined to achieve clinical significance [11].

BODY: We hypothesize that benign and malignant prostatic disease will each produce proteomic changes in circulating leukocytes characteristic of their disease. By analyzing the proteomes of leukocytes, we will be able to discriminate between the two classes of disease non-invasively. We furthermore wish to utilize the observed proteomic differences to develop a test that could be used when an individual is observed to have an elevated serum level of PSA, in order to distinguish among CP, BPH and PrCA. To achieve these objectives, we proposed to employ mass spectrometry to produce comprehensive proteomic profiles of peripheral leukocytes from 100 individuals with elevated PSA, and correlate the results with those obtained by histologic examination of core-biopsy samples. Progress towards these goals are detailed below.

Issues Identified In Year One Progress Report

1. Patient recruitment/sample acquisition

From our Year 1 progress report, we stated:

> "The key task to be confronted in year 2 is an accelerated enrollment of clinical samples. There is a sufficiently ample case load at Boston University Medical Center for this study, but the clinical personnel are so burdened by oversubscribed clinical schedules that the slight, but additional, time involved with patient recruitment has proved challenging. While the budget does not allow for a clinical coordinator that could separately handle this task, an increased role will be taken by the PI (Steffen) in presenting the details of the study to patients and consenting the willing participants."

This issue has been addressed and largely ameliorated. A revised patient recruitment plan was developed. The plan has three major new components: (1) Patients are identified in advance by Urology scheduling staff, and a list is forwarded to the PI Steffen for the coming week. The PI then calls the patient before their appointment explaining they are eligible for participation in a research project concerning prostate disease, and if they are interested in learning more about the project, and potentially participating, they are to arrive one hour early for their appointment. (2) The PI Steffen then meets each patient at the Urology clinic, introduces the project to the patient, and if the patient is interested in participating, consents each patient. D3) Patients are offered a voucher for $25 for participating in the study. This has increased the patient participation rate from <10% to approximately 50% of patients that are identified and contacted in advance.

The new plan was submitted to and approved by the Boston University Institutional Review Board. The Boston University approved was submitted to the Human Research Protection Office (HRPO) of the USAMRMC and approved on May 4, 2011. After the mechanism for paying the patients was established (patients are given a voucher which they present to the hospital cashier), patient recruitment and sample acquisition under the new protocol began in June. We have averaged two new samples per week of sample collection.

2. Issues Related To Cell Integrity In Aim 2

From our Year 1 progress report, we stated:

> "Proteomic profiles of leukocytes following in vitro exposure of leukocytes to tumor cell lines - has encountered issues related to cell viability following acquisition from a commercial source. The main objective for this Aim was the elimination of genetic variation observed in samples from any patient cohort in order to identify differences solely due to the presence of the tumor… Whether any of these constitute a change to the original Statement of Work will be discussed in short order with the Army Contracting Officer Representative, and if so, approval will be obtained."

After an exploration of alternatives to address this problem, we elected to pursue a strategy of comparing leukocytes in a mouse model of prostate cancer to a genetically similar mouse that does not develop prostate cancer (details below). Following a discussion with Dr. Mishra, the PCRP Science Officer for our project, we concluded that a new SOW should be submitted.

In Aim 2, we originally proposed an *in vitro* test "allowing a direct comparison of single-source leukocytes in the presence or absence of tumor cells." We acknowledged the shortcomings of an *in vitro* experiment, but Scientist Reviewer A made this comment: "it provides a simplified model that minimizes the heterogeneity that will likely be present in patient samples." Our proposed change was to compare the leukocytes in a mouse model of prostate cancer. This retains the advantage of a "simplified

model that minimizes the heterogeneity that will likely be present in patient samples" while switching from "*in vitro*" to "*in vivo*" makes the findings much more germane to eventual clinical translation.

Our change proposes to achieve the same goal, but by an improved method, that has more direct relevance to human health, and will likely improve the probability of translating our findings to the clinic. The goal of Aim 2 was to compare the same immune system in the presence or absence of prostate cancer cells in order to identify changes that are specific to the cancer, and not related to genetic heterogeneity, such as will be encountered in any human cohort. The original proposal was an *in vitro* study in which single source leukocytes would be split in two, and incubated on top of cultured cell lines of prostate cancer or prostate epithelium. We now have proposed and *in vivo* study looking at genetically identical leukocytes in isogenic mice strains, one of which develops prostate cancer in response to tissue-specific transgene expression.

The mouse which develops prostate cancer is referred to as the “TRAMP” model, short for “TRansgenic Adenocarcinoma of the Mouse Prostate,” and has the strain designation of "C57BL/6-Tg(TRAMP)8247Ng/J," distributed by Jackson labs. The control strain is the frequently used "C57BL/6" strain.

Three developments contributed to our decision to utilize this model. (1) From the very first 2007 submission, PCRP reviewers commented for this very aim that. “A weakness is the unavoidably contrived in vitro conditions that may permit cell-cell interactions that do not occur in vivo, leading to discovery of false positives.” (2) I presented a progress report of our results on my prostate Cancer project to the Boston University Hormone-responsive Cancer Program. After the presentation there was a discussion about our work, and a number of senior investigators suggested that I look into the mouse model as perhaps being more relevant for our ultimate goal, which are human studies. (3) At the IMPaCT 2011 conference in Orlando, Florida, one of the keynote speakers, Eugene Kwon of the Mayo clinic presented his work on the TRAMP mouse model mentioning its utility for his studies on the interaction between the immune system and prostate cancer in mice.

Following the IMPaCT meeting, we developed, and our Office of Sponsored Programs then submitted a new SOW, which was approved. An animal protocol for pursuing the studies was developed, and reviewed and approved by the Boston University IACUC committee. The IACUC approval letter and the animal protocol was forwarded to the Animal Care and Use Review Office (ACURO) and approved. Because of the delays associated with getting the animal protocols approved, and that once we are able to place the order for the TRAMP mice, it takes several months for them to develop metastatic prostate cancer, we requested a four-month no-cost extension to complete the animal studies. For these mice, metastatic tumors become palpable at 10-38 weeks, at which point they will be sacrificed, with blood and tissues harvested. We will take possession of the mice in January 2012.

Co-Isolation Of Monocytes and $CD4^+$ T-lymphocytes

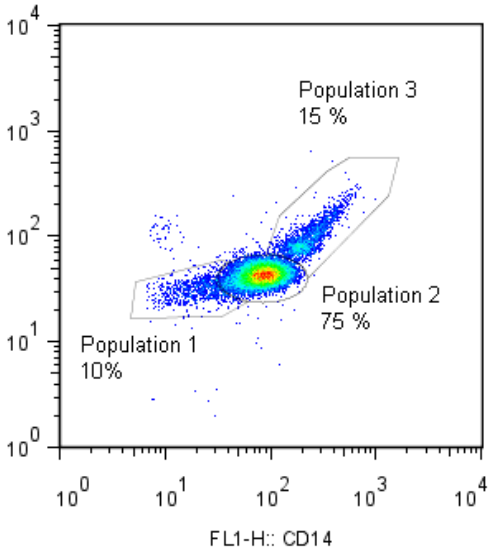
From preliminary data and consideration of known biology, we were especially eager to establish a protocol for the isolation of both monocytes and $CD4^+$ T-lymphocytes in high numbers, with high purity, and high reproducibility from the same patient. High cell number is important to ensure sufficient proteomic material for mass spec analysis. We are limited by our IRB protocol and the accepted definition of minimal risk to collecting no more than 49 mls of blood from a patient. Previously, to achieve high purity for two cell populations, we would split samples into two aliquots and isolate one cell type from each aliquot. Occasionally, this did not permit the isolation of a sufficient number of cells for optimal mass spec analysis. A number of the approaches were explored, and in the end, got the best results when we isolated PBMCs by buoyant density separation, performed positive selection of monocytes using CD 14+ beads (Miltenyi), and then employed a negative selection for $CD4^+$ T-lymphocytes on the flow-through material. From this procedure we are able to reproducibly isolate high cell numbers of high percent purity of the desired cell type.

	Purity (%)
Monocytes isolated by positive selection from PBMCs	97.9 $\pm$ 0.3
CD4+ cells isolated with negative selection on supernatant after isolation of monocytes	97.2 $\pm$ 1.0

Table 1. Purity of monocytes and CD4+ T- lymphocytes, as measured by FACS analysis. The average and standard deviation is calculated from 11 patient samples.

One concern associated with positive selection of peripheral monocytes, heterogeneous to begin with, is the alteration of subpopulation percentages, in particular the activation of monocytes along pathways leading to mature macrophages. We have examined this, and conclude that this is not a major confounding variable for our experiments. In figure 1, we show FACS analysis of our purified monocytes, showing three subpopulations.

Figure 1. Examination of monocyte subpopulations using fluorescent CD14 antibodies. Three populations are clearly observed. From the literature [1,2], sub-population 3 monocytes differentiated into macrophages. Sub-populations 1 and 2 are referred to as resident and inflammatory monocytes. The relative sub-population percentages are listed in Table 2. The amount of cells in sub-population 3 can, and have, been obtained using only forward- and side- scatter, so as not to require an additional fluorescent antibody which might also induce subpopulation changes.



In Table 2, we show the relative amounts of the three subpopulations are not differing in our cancer versus control samples, based on analysis of nine samples.

	Monocyte Sub-Population Percentages After Positive Selection		
	Cancer	**Control**	**All**
Population 1	9.9 ± 2.8	9.4 ± 1.1	9.6 ± 2.4
Population 2	73.8 ± 7.4	74.1 ± 3.1	73.9 ± 6.3
Population 3	14 ± 2.6	14.4 ± 2.9	14.2 ± 2.9

Table 2. FACS analysis showing that positive selection is not significantly altering monocytes subpopulations differentially in cancer and control samples. Numbers represent averages from 5 cancers and 4 non-malignant prostate conditions.

Mass Spectrometric Analysis of Isolated Monocytes

Protein from about 1.5 million monocytes (60 ug) are separated on a 10 cm 4-20% polyacrylamide gel (Invitrogen). The entire gel lane was cut into 20 sections, and subjected to reduction with DTT, alkylation with iodoacetamide and manual in-gel tryptic digestion. The extracted tryptic peptides are collected in a 96 well plate, dried and re-suspended in 0.5% acetic acid The samples are loaded sequentially onto reverse phase Magic C18 columns (100 micron X 10 cm) using a ThermoAS auto-sampler. The peptides are separated using 30 minute gradients of 5-90% acetonitrile gradient in the presence of 0.5% of acetic acid. The nanospray column is directly interfaced to the orifice of a ThermoFinnigan LTQ ProteomeX ion trap mass spectrometer. Tandem mass spectra are collected for the top ten ions of each MS scan. The tandem mass spectra are analyzed using SEQUEST.

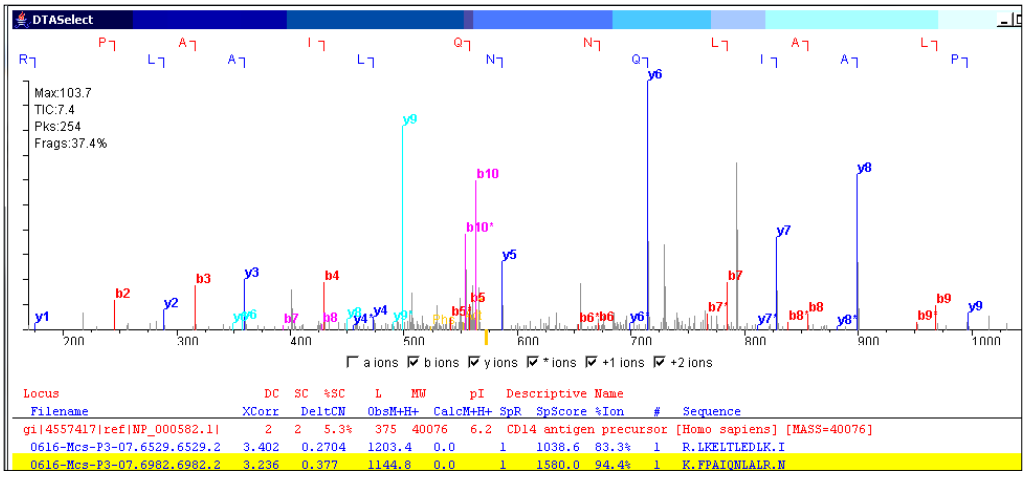


Fig. 2. MS2 scan identifying the peptide FPAIQNLALR from the protein CD14, the protein which is used to define, and purify the monocytes.

Spectra were queried against NCBI RefSeq protein database. Proteins were considered present only if two high-scoring unique sequence peptide matches were achieved in a single gel slice or if a single peptide was observed at two different charge states in that gel slice. The XCorr identification thresholds were adjusted such that the false positive rate of protein identification, determined empirically by the addition of decoy peptides, was 2%. Fig. 2 shows a scan identifying a peptide from the protein PTGES3, a protein that appears to be noticeably upregulated in CD4+ T-lymphocytes of patients with elevated serum PSA and the diagnosis of prostate cancer.

From an analysis of 10 samples, we are identifying an average of 534 proteins with two or more unique peptides, and an empirically determined false positive rate of 2% (standard deviation $\pm$ 70 proteins). A total of 1190 proteins were observed in at least one sample, and 843 proteins in at least two samples. Of particular interest are the set of CD antigens, which are frequently functionally important, and used to define leukocyte subgroups, which may have important functional consequences. We observe a total of 12 CD antigens: CD14, CD33, CD36, CD44, CD47, CD48, CD63, CD68, CD74, CD84, CD93, CD97, CD163. Five of these (CD33, CD36, CD68, CD74, and CD163), have a preliminary indication that they may be differentially expressed in samples from patients with prostate cancer.

Protein ID	Avg Counts in Cancer Samples	Avg Counts in Control Samples	*p* val	Expression Higher in Cancer Samples	Expression Higher in Control Samples	Description
NP_001011.1	3.0	0.0	0.04	x		ribosomal protein S16
NP_009029.2	7.2	0.0	0.05	x		clathrin, heavy polypeptide-like 1
NP_001003.1	6.6	1.1	0.08	x		ribosomal protein S8
NP_004346.1	5.1	0.9	0.01	x		CD74 antigen isoform b
NP_002958.2	1.1	6.8	0.01		x	scaffold attachment factor B
NP_057417.3	0.0	3.0	0.01		x	splicing coactivator subunit SRm300
NP_004235.3	0.0	4.1	0.01		x	CD163 antigen isoform a
NP_004951.1	0.0	2.5	0.06		x	fusion, t(12;16) in malignant liposarcoma
NP_055629.1	0.0	1.8	0.06		x	proteasome 26S subunit, non-ATPase, 6
NP_001307.2	0.0	3.8	0.06		x	CSE1 chromosome segregation 1-like protein
NP_002363.2	0.0	1.0	0.06		x	mannosidase, alpha, class 2A, member 1
NP_061916.3	0.0	3.8	0.06		x	amyloid beta (A4) precursor protein-binding
XP_001720895	0.0	3.3	0.06		x	PREDICTED: hypothetical protein isoform 3
NP_036533.2	0.0	4.3	0.07		x	plexin B2
NP_002712.1	0.0	1.3	0.07		x	protein phosphatase 6, catalytic subunit
NP_002795.2	0.0	1.3	0.07		x	proteasome 26S ATPase subunit 3
NP_061899.1	0.0	2.0	0.07		x	transmembrane and coiled-coil domains 1
NP_003096.1	0.0	2.8	0.08		x	sortilin-related receptor, LDLR class A repeats
NP_079199.2	0.0	6.5	0.09		x	nucleoporin 210

Table 3. High abundance proteins that, at an early stage, indicate potential as class discriminators. Protein abundance is estimated by spectral counts [3]. Proteins whose expression in monocytes is elevated in prostate cancer are shaded in red. Proteins whose expression is elevated in controls is shaded in green. The *p* values should be regarded as preliminary, and are included only to serve as a guide, on account of the relatively small number of samples at this point.

Protein ID	Percent Cancer Samples	Percent Control Samples	Expression Higher in Cancer	Expression Higher in Control	Description
NP_062826.2	67	25	x		methyltransferase like 3
NP_076997.1	67	25	x		transmembrane protein 109
NP_056937.2	67	25	x		UBX domain protein 1
NP_000996.2	67	25	x		ribosomal protein S3
NP_149347.1	67	25	x		caldesmon 1 isoform 3
NP_665875.1	67	25	x		annexin A11
NP_003818.2	67	25	x		N-ethylmaleimide-sensitive factor
NP_001782.1	67	25	x		cell division cycle 42 isoform 1
NP_004108.1	67	25	x		FK506 binding protein 5
NP_056284.1	67	25	x		nipsnap homolog 3A
NP_004823.1	67	25	x		glutathione-S-transferase omega 1
NP_001763.3	67	25	x		CD33 antigen isoform 1 precursor
NP_005137.1	50	0	x		squamous cell carcinoma antigen 1
NP_663782.1	50	0	x		adaptor-related protein complex 1 beta 1
NP_006001.2	50	0	x		arginine-rich, mutated in early stage tumors
NP_055951.2	50	0	x		stabilin 1 precursor
NP_055135.1	50	0	x		heme binding protein 2
NP_005156.1	50	0	x		fructose-bisphosphate aldolase C
NP_001147.1	50	0	x		annexin VII isoform 1
NP_942126.1	50	0	x		ras-related C3 botulinum toxin substrate 1
NP_060846.2	50	0	x		nipsnap homolog 3B
NP_000398.1	50	0	x		glycoprotein Ib, beta polypeptide precursor
NP_002718.2	0	50		x	serglycin precursor
NP_056992.4	0	50		x	arsenate resistance protein 2 isoform a
NP_00110822	0	50		x	decay accelerating factor for complement
NP_001242.2	0	50		x	CD68 antigen isoform A

Table 2. Low abundance proteins that, at an early stage, indicate potential as class discriminators. Protein abundance is estimated by the observed presence or absence of a protein within a sample. Proteins whose expression in monocytes is elevated in prostate cancer are shaded in red. Proteins whose expression is elevated in controls is shaded in green.

2D Gel Electrophoresis. We were intrigued about 2D gel methodology last year, but common concerns about the reproducibility of 2D gels have borne out, and we do not project that we will aggressively pursue this avenue in the coming year, unless we gain access to a Typhoon imager (GE Healthcare) which would permit DIGE, two-color experiments on the same gel.

KEY RESEARCH ACCOMPLISHMENTS:

- Revised patient recruitment protocol to increase sample acquisition rate.
- Initiated revised Aim 2 which will examine genetically identical monocytes in the presence or absence of prostate cancer in an *in vivo* model.
- Have established high standards of purity and sample yields for the co-isolation of monocytes and $CD4^+$ T-lymphocytes from a single patient sample.
- Have analyzed clinical sample proteomes from monocytes using shotgun mass spectrometry.

REPORTABLE OUTCOMES:

1.) Presented poster: "Proteomics Of Peripheral Leukocytes In Patients With Elevated Serum Levels Of Prostate Specific Antigen." Martin Steffen, Agnes Bergerat, Mark Katz, David Wang, and Richard Babayan. Innovative Minds in Prostate Cancer Today (IMPaCT) Conference , March 9–12, 2011. Orlando, Florida

2.) Two grant applications were submitted that builds on the methods developed in years 1-2 here.

NIH - "A test to diagnose pediatric asthma," R21, (Steffen - PI)

American Asthma Foundation Research Program - "Development of a Definitive Blood Test for the Diagnosis of Asthma in Young Children" (Steffen - PI)

CONCLUSION: We remain enthusiastic about the prospects for identifying molecular differences in circulating leukocytes for patients with prostate cancer, which will be clinically useful for reducing the number of biopsies performed. The two major obstacles encountered last year, the rate of patient enrollment, and the difficulties associated with characterizing single-source monocytes in the presence and absence of prostate cancer, have been overcome. Proteins that are differentially expressed in our mouse model and control strain will become targets to validate our human populations. Proteins can be validated with mass spectrometry and antibody-based methods, allowing for high confidence in any observed differences. If the differences are robust, it will be appropriate to evaluate the observations in wider clinical populations, ideally such as the Prostate Cancer Clinical Trials Consortium (PCCTC). If one can increase the certainty that an elevated PSA is a result of prostate cancer, and not due to nonmalignant prostate disease, one could pursue evaluating lower PSA levels as indicators of cancer, to increase the sensitivity of the test. This of course is documented in the literature, but it is always concerns about the higher false positive rates that lower levels of PSA are not considered suitable for routine clinical use.

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APPENDICES: N/A